Regional difference in the distribution of L-NAME-sensitive and -insensitive NANC relaxations in cat airway

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1. To investigate the distribution profile of functional inhibitory non-adrenergic non-cholinergic (i-NANC) nerves and the contribution of NO to the NANC relaxation in the cat, we studied the effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on NANC relaxation elicited by electrical field stimulation (EFS) in the trachea, bronchus and bronchiole.

2. EFS applied to the tracheal smooth muscle during contraction induced by 5-HT (10^{-5} M) in the presence of atropine (10^{-6} M) and guanethidine (10^{-6} M) elicited a monophasic NANC relaxation. By contrast, NANC relaxation elicited in the peripheral airway was biphasic, comprising an initial fast followed by a second slow component and L-NAME (10^{-5} M) selectively abolished the first component without affecting the second one. In the trachea, L-NAME (10^{-5} M) completely suppressed the monophasic NANC relaxation when single or short repetitive stimuli (< 5) with 1 ms pulse duration were applied. However, at higher repetitive stimuli (> 10) with 1 or 4 ms pulse duration, suppression of NANC relaxation was incomplete.

3. In the small bronchi obtained from L-NAME-pretreated cats, EFS applied during contraction induced by 5-HT (10^{-5} M) elicited only the slow component of NANC relaxation which is sensitive to tetrodotoxin.

4. In the peripheral airway, a newly synthesized VIP antagonist (10^{-6} M) or α-chymotrypsin (1 U ml^{-1}) considerably attenuated the amplitude of L-NAME -insensitive relaxation.

5. Single or repetitive EFS consistently evoked excitatory junction potentials (EJPs) in the central and peripheral airways. When tissues were exposed to atropine (10^{-6} M) and guanethidine (10^{-6} M), single or repetitive EFS did not alter the resting membrane potential.

6. These results indicate that at least two neurotransmitters, possibly NO or NO-containing compounds and VIP, are involved in i-NANC neurotransmission and the distribution profile of the two components differs in the central and peripheral airway of the cat.

The tracheobronchial smooth muscle is innervated by nerve fibres from cranial parasympathetic outflow and sympathetic trunks (Smith & Taylor, 1971). Recent studies revealed that the cranial parasympathetic nervous system in the airway contains non-adrenergic non-cholinergic (NANC) inhibitory nerves in addition to the well-documented cholinergic excitatory nerve fibres. Furthermore, activation of C-fibre afferent (sensory) nerves induces a number of airway responses including smooth muscle contraction, mucus secretion and plasma exudation through NANC excitatory transmitter (Barnes, 1991). Thus, at least two functional inhibitory and excitatory nervous systems have been demonstrated in airway smooth muscle, adrenergic, cholinergic and inhibitory and excitatory NANC (i- and e-NANC) nervous systems.

In contrast to the dense cholinergic innervation throughout the tracheobronchial tree of all mammals, it is generally considered that the presence and distribution of the inhibitory nervous systems vary between species and regions of the airway. For example, functional adrenergic nerves are present throughout the airways of dogs (Russel, 1980), but are absent in human bronchi (Richardson & Beland, 1976). The i-NANC nervous system was demonstrated in various animal species including guinea-pig (Coburn & Tomita, 1973), cat (Ito & Takeda, 1982), pig (Mitchell, Sparrow & Taglaferri, 1990), baboon (Middendolf

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& Russell, 1978) and human (Richardson & Beland, 1976), but is absent in canine airways (Ito & Tajima, 1981). In guinea-pigs, adrenergic and i-NANC innervations have been demonstrated in the trachea (Ellis & Undem, 1990), and recent reports indicate that i-NANC nerves only supply the trachea and central bronchi, but not the peripheral bronchi in equine airway (Yu, Wang, Robinson & Leblanc, 1994).

The neurotransmitters responsible for the NANC relaxation in the airways have not been conclusively identified; however, NO and vasoactive intestinal polypeptide (VIP) are the candidates for this role (Palmer, Cuss & Barnes 1986; Li & Rand, 1991; Belvisi *et al.* 1992; Jing, Inoue, Tashiro, Takahashi & Ito, 1995).

In human airways, for example, it was reported that the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) produced a concentration-dependent inhibition of the i-NANC relaxation, producing almost complete inhibition at every frequency of electrical field stimulation (EFS) employed (0·5–40 Hz; Belvisi *et al.* 1992). Furthrmore, α-chymotrypsin did abolish the relaxation induced by exogenously applied VIP but not the NANC relaxation evoked by EFS. Thus, the authors concluded that in human airways i-NANC relaxation is mediated entirely by NO, although VIP-like immunoreactive nerves are present in the smooth muscle layer of the respiratory tract (Laitinen, Partanen, Hervonen, Pelto-Huikko & Laitinen, 1985).

On the other hand, in the guinea-pig trachea, NO synthase inhibitors only partially suppressed the NANC relaxation (Tucker, Brave, Charalambous, Hobbs & Gibson, 1990; Li & Rand, 1991) and a significant proportion of the remainder of the relaxation was substantially suppressed by α-chymotrypsin (Ellis & Farmer 1989*a*, *b*; Li & Rand, 1991) or incubation with antisera against VIP or peptide histidine isoleucine (PHI; Matsuzaki, Hamasaki & Said, 1980; Ellis & Farmer 1989*b*). These observations indicate that in the guinea-pig trachea, peptides such as VIP or PHI and NO are involved in the NANC relaxations.

In contrast, in feline airways, conflicting results have been reported; in one study it was reported that NO did not mediate the NANC relaxation *in vivo* or *in vitro* (Diamond, Lantta, Thompson & Altiere, 1992), while in another study, NO was proposed as the primary, if not only, mediator for the NANC relaxation (Fisher, Andersen & Waldron, 1993). Furthermore, our recent studies indicate that NANC relaxation can be classified into two different components according to the sensitivity to NO synthase inhibitors or to the threshold for activation, suggesting that at least two different neurotransmitters are involved in the NANC relaxation (Jing *et al.* 1995). These experiments, however, have been carried out using trachea, and there are no reports describing NANC inhibitory nervous system in the peripheral (bronchi and bronchioles) airways in this animal.

The present experiments were carried out to examine firstly, the distribution profile of functional i-NANC nerves in the cat airway, and secondly, whether, and to what extent, NO mediates the NANC relaxation in the different regions of cat airway. In the present experiments we used cervical trachea, segmental bronchus, small bronchus and bronchiole of cat airways.

METHODS

Adult mongrel cats of either sex (2–3 kg) were anaesthetized with sodium pentobarbitone (30–40 mg kg^{-1}, I.P.) and then bled. In some experiments, cats were pretreated with L-NAME (1 mmol kg^{-1}) administered I.P. for 2 days daily, or L-NAME was given to cats for 1 week in drinking water, before they were killed. Segments of cervical trachea and whole pulmonary lobes were quickly resected from the main bronchus. A dorsal strip of transversely running tracheal smooth muscle was separated from the cartilage and the mucosa and adventitial alveolar tissue were carefully removed. The mucosa from the tracheal muscle was removed, leaving only the smooth muscle. The tracheal smooth muscle was cut to a width of 2–3 mm and a length of about 5 mm for recording of mechanical responses. We also used bronchi and bronchioles and classified bronchi into two categories, namely segmental branch of lobar bronchi (3–5 mm o.d.) and small bronchi (1–3 mm o.d.). Since the branching pattern in the cat airway is not symmetrical and regular as in the case of humans (Mortensen, Young, Strout, Strout, Bagley & Schaap, 1983), but is similar to that of dog (Amis & McKiernan, 1987), the diameter of bronchi does not necessarily correlate with the order of branching of the bronchi. The diameters of segmental bronchi of lobar bronchi in each lobe were in the range of 3–5 mm i.d., and bronchioles (> 1 mm) could be easily identified by the lack of cartilage through microscopic observations. Bronchi with i.d. 1–3 mm were classified as small bronchi. The small airways (about 1–5 mm i.d.) were carefully excised from the lung tissue under microscopic observation, and lung parenchyma and pulmonary vessels running along a bronchiolar branch were carefully removed under microscopic observation. Concerning bronchioles, histological investigations confirmed that the tissue used for the present experiments had an i.d. of 0·8–1·1 mm and was composed of smooth muscle layers and mucous membrane but lacked cartilage, thereby indicating that the tissue comprised bronchioles (Cumming, 1972; Ito & Inoue, 1989). Segments of bronchi and bronchioles dissected free of the lung parenchyma and prominent surface vessels were cut into rings 2–3 mm wide. Airway epithelium was carefully removed as much as possible by mechanical rubbing according to the method described elsewhere (Xie, Hakoda & Ito, 1992), since it is known that EFS stimulates the airway epithelial cells to release factor(s) which induce the relaxation of dog bronchioles in the presence of indomethacin, atropine and guanethidine and when they have been precontracted with 5-HT (10^{-5} M; Xie, Hakoda & Ito, 1992). The preparation was bathed in a modified Krebs solution of the following ionic concentrations (mM): 137·4 Na^+, 5·9 K^+, 1·2 Mg^{2+}, 2·5 Ca^{2+}, 134·0 Cl^-, 1·2 $H_2PO_4^-$, 15·5 HCO_3^- and 11·5 glucose. The solution was aerated with 97 % O_2 and 3 % CO_2 and the pH was 7·3–7·4.

For measurement of mechanical responses, the tracheal tissue was mounted vertically in a 1 ml organ bath through which the test solution, at 35–36 °C, flowed continuously at a rate of 3 ml min^{-1}. One end of the strip was tied by fine silk thread to a mechanotransducer (RCA-5734, Nihon Kohden Ltd, Tokyo) and

the other to a hook at the bottom of the bath. The strips were set up with an initial tension of 0·1–0·2 g and mechanical activity was recorded with a pen recorder. To measure the mechanical responses of the ring preparations of bronchi and bronchioles, the preparations were hooked horizontally in a 1 ml organ bath through which the test solution flowed continuously at a rate of 2 ml min^{-1}, by a pair of right-angled fine needles which were reduced in diameter by electrolysis to about 50 μm as observed under a microscope. One needle was fixed to the wall of the chamber. The other needle was connected to a manipulator at one end and at the other end to an isometric tension transducer, through a 1 mm wide slit made on the other wall of the chamber. EFS was applied through a pair of Ag–AgCl plates fixed to both sides of the inner surface of the chamber, so that current pulse would pass transversely across the ring preparations of the bronchi and bronchioles.

For single cell intracellular recording of membrane potential, ring preparations of bronchi and bronchioles (3–7 mm in length) with intact inner lumen were used. A conventional glass microelectrode filled with KCl (3 M, 30–50 MΩ) was inserted from the outer surface. Field stimulation was applied to the nerves through a pair of Ag–AgCl wires (3–5 mm apart) placed so that a current pulse would pass transversely across the tissue. Single and repetitive stimuli were applied at 20 Hz, with a pulse of 50 μs duration and 20–30 V strength using an electronic stimulator (Nihon Kohden, SEN-7103). The chamber in which the strips were mounted had a volume of 2 ml and was superfused at a rate of 3 ml min^{-1} at 35–36 °C. To avoid recording artifacts due to twitch-like contraction of the muscle tissue, the preparation was pinned to the rubber plate in the chamber using insect pins with 100 μm diameter.

The following drugs were used : N^G-nitro-L-arginine (L-NNA), L-NAME, isoprenaline-bitartrate (Nakarai Chemicals, Kyoto, Japan), 5-hydroxytryptamine hydrochloride (5-HT), L-isoprenaline hydrochloride and acetylcholine chloride (Sigma), guanethidine monosulphate (Tokyo Kasei, Tokyo), VIP antagonist (Peninsula Laboratories, Inc), atropine sulphate (Daiichi, Tokyo), tetrodotoxin (Sankyo, Tokyo) and α-chymotrypsin (Nakarai Chemicals, Kyoto, Japan). The drugs were added to the perfusing solution. Concentrations of drugs in text refer to their salts.

Results (amplitude of muscle relaxations and resting membrane potential) are expressed as means ± S.D. and were analysed for statistical significance by Student's t test. Significance was indicated where $P < 0·05$.

RESULTS

Effects of L-NAME on EFS-induced NANC relaxation in central and peripheral airways

In the cat trachea, EFS (repetitive stimuli at 20 Hz) applied during contraction evoked by 5-HT (10^{-5} M) in the presence of atropine and guanethidine (10^{-6} M each) consistently produced phasic relaxation and when the duration of the stimulus pulse or the number of stimuli was

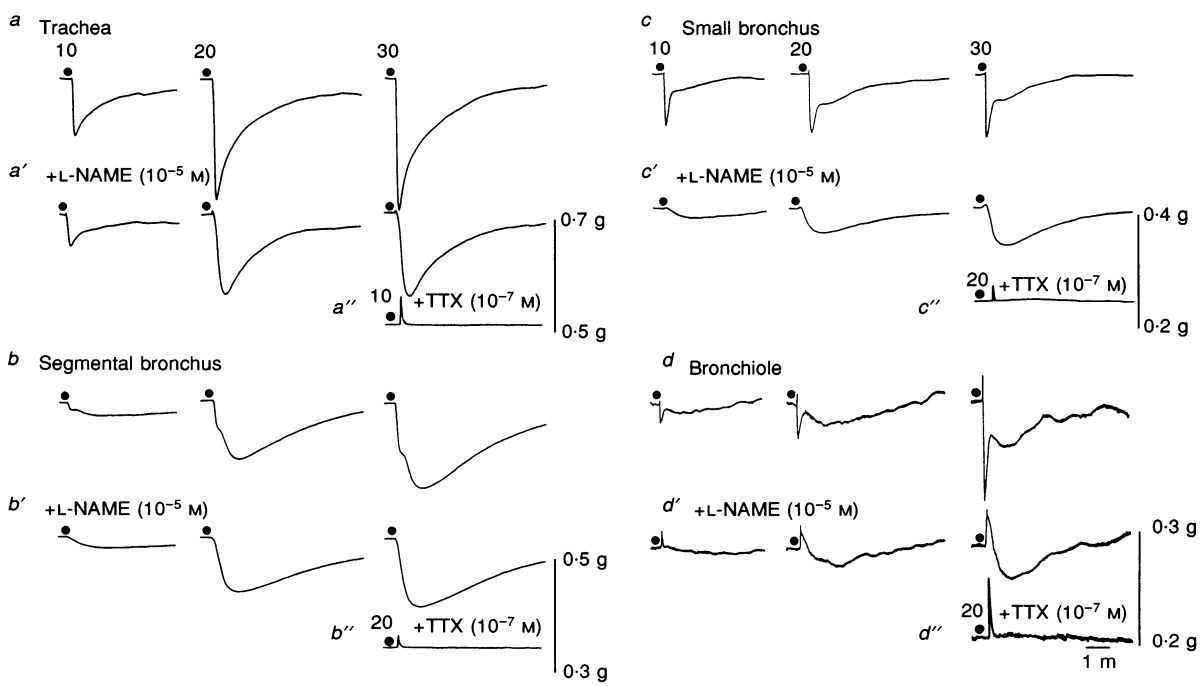

Figure 1. Effects of L-NAME (10^{-5} M) on EFS-induced NANC relaxations in central and peripheral airway

a–d, effects of L-NAME (10^{-5} M) on EFS-induced NANC relaxations in the presence of atropine, guanethidine (10^{-6} M each) and 5-HT (10^{-5} M). Repetitive field stimulation of 10, 20 and 30 stimuli at 20 Hz with 4 ms pulse duration (●) during the contraction evoked by 5-HT-induced NANC relaxation in the absence (*a–d*) and presence of L-NAME (10^{-5} M; *a′–d′*), in trachea, segmental bronchus, small bronchus and bronchiole. *a″–d″* show effects of TTX (10^{-7} M) on NANC relaxation in the presence of L-NAME.

increased in a stepwise manner at a constant stimulus intensity and frequency (20 Hz), the amplitude of the relaxation increased proportionally. Furthermore, we noticed that L-NNA or L-NAME did not abolish the NANC relaxation, when EFS with 1 or 4 ms pulse duration and over ten repetitive stimuli at 20 Hz was applied, and a substantial part of the remainder of the NANC relaxation is tetrodotoxin sensitive (Jing *et al.* 1995). Therefore, we applied repetitive field stimulation with 1 and 4 ms at 20 Hz to the tracheal strips and ring preparations of segmental bronchi, small bronchi and bronchioles. EFS consistently evoked NANC relaxation in all the preparations examined, in the presence of atropine and guanethidine, and when precontracted with 5-HT (10^{-5} M). Figure 1 shows an example of EFS-induced NANC relaxation in the presence or absence of L-NAME (10^{-5} M), where ten, twenty and thirty stimuli at 20 Hz with 4 ms pulse duration were applied. In the tracheal strips, EFS induced monophasic relaxation, the amplitude of which increased in proportion to the number of stimuli. On the other hand, in the segmental bronchi, a clear 'hump' during the course of NANC relaxation was observed within several seconds after the application of EFS. Furthermore, EFS induced clear biphasic NANC relaxations in small bronchi and bronchioles. Namely, EFS evoked initial peak relaxation 7·4 ± 2·2 s (± S.D., $n = 23$, ranging between 3·2 and 9·6 s) after the application of EFS, which was followed by a second component of NANC relaxation with a slower time course. The mean time to peak relaxation of the second component after EFS and measured in the presence of L-NAME (10^{-5} M) was 77·6 ± 20·3 s (± S.D., $n = 17$, ranging between 54 and 132 s). These NANC relaxations were completely abolished by tetrodotoxin (10^{-7} M), thereby indicating their neurogenic origin.

The relaxation response to repetitive stimuli (30 stimuli at 20 Hz) with 4 ms pulse duration was not significantly different in all of the regions examined, although there was a tendency for the relative amplitude of NANC relaxation to diminish along the central to peripheral airways (Fig. 2).

L-NAME (10^{-5} M for 30 min) markedly reduced the NANC relaxation in tracheal strips, abolished the 'humps' in the segmental bronchi and initial peak relaxations in the small bronchi and bronchioles. Increased concentration of L-NAME (10^{-4} M) or L-NNA (10^{-4} M) did not further reduce the amplitude of the L-NAME (10^{-5} M)-resistant tracheal relaxation or the amplitude of the second component of the NANC relaxations in the small bronchi and bronchioles (data not shown). Figure 3*A* shows effects of L-NAME (10^{-5} M) on the relationship between the number of stimuli at 20 Hz and the relative amplitude of the NANC relaxation observed in trachea, segmental bronchi, small bronchi and bronchioles, where the amplitude of NANC relaxation induced by thirty stimuli with 4 ms pulse duration was taken as a relative relaxation of 1·0. In the trachea, L-NAME (10^{-5} M) almost completely suppressed the relaxation evoked by ten stimuli with 1 ms pulse duration. However L-NAME (10^{-5} M) did not abolish the NANC relaxation when EFS with 1 or 4 ms pulse duration and over ten repetitive stimuli at 20 Hz were applied. In contrast, in the segmental and small bronchi or bronchioles, L-NAME did not abolish the NANC relaxation even when EFS with less than ten stimuli with 1 ms pulse duration was applied. There is a tendency that the smaller the airway, the larger the amplitude of L-NAME-insensitive NANC relaxation especially when a longer stimulus pulse (4 ms) was used. Figure 3*B* shows the relative amplitude of L-NAME (10^{-5} M)-insensitive NANC relaxation in various regions of cat airway when thirty stimuli at 20 Hz with 1 and 4 ms pulse duration were applied, and the amplitude of the maximum relaxation evoked by thirty stimuli with 4 ms pulse duration was taken as a relative relaxation of 1·0. These observations indicate that two different components are involved in the NANC relaxation evoked by repetitive EFS with short or long pulse duration, and that the L-NAME-insensitive component is dominant in the peripheral airways.

To examine whether it is possible to distinguish the two components according to the threshold for activation, we used the ring preparations of the bronchioles. Figure 4*Aa* shows examples of the effects of single or repetitive stimuli at 20 Hz with 600 μs pulse duration at 50 V intensity. Single or repetitive stimuli (5 stimuli) at 20 Hz evoked only the initial fast NANC relaxation, and the peak of the relaxation was obtained 3·4 ± 0·2 s (± S.D., 6 trials) after the end of EFS in this particular example. When the

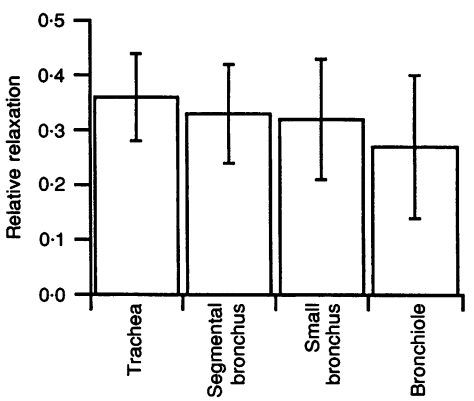

Figure 2. Relative amplitude of NANC relaxation in central and peripheral airway

Relative amplitude of NANC relaxation in trachea, segmental bronchus, small bronchus and bronchiole. Relative amplitude of NANC relaxations evoked by repetitive field stimulation of 30 stimuli at 20 Hz with 4 ms in the central and peripheral airways, where muscle relaxation evoked by 10^{-8} M isoprenaline in the presence of atropine, guanethidine (10^{-6} M each) and 5-HT (10^{-5} M) was taken as a relative relaxation of 1·0. Bars indicate 2 × S.D.

number of repetitive stimuli at 20 Hz was increased (> 10) in a stepwise manner, EFS evoked an initial fast NANC relaxation followed by a second NANC relaxation with slower time course. Figure 4*Aa'* shows the effects of L-NAME (10^{-5} M) on the NANC relaxations; L-NAME selectively abolished the initial component without affecting the second component of the NANC relaxations. The inhibitory effects of L-NAME (10^{-5} M) on the initial component was partially restored (to 0·6–0·8 of the original amplitude) by L-arginine (10^{-4} M) but not D-arginine (10^{-4} M; data not shown). Time to peak of the second component after EFS was 82·5 ± 1·8 s (± S.D., 5 trials) and lasted for 2–4 min after the stimulation, indicating that the time course of the second component is much slower than that of the initial component.

These observations indicate that the threshold for the activation of the initial and second component of the NANC relaxation is different, and the threshold for the

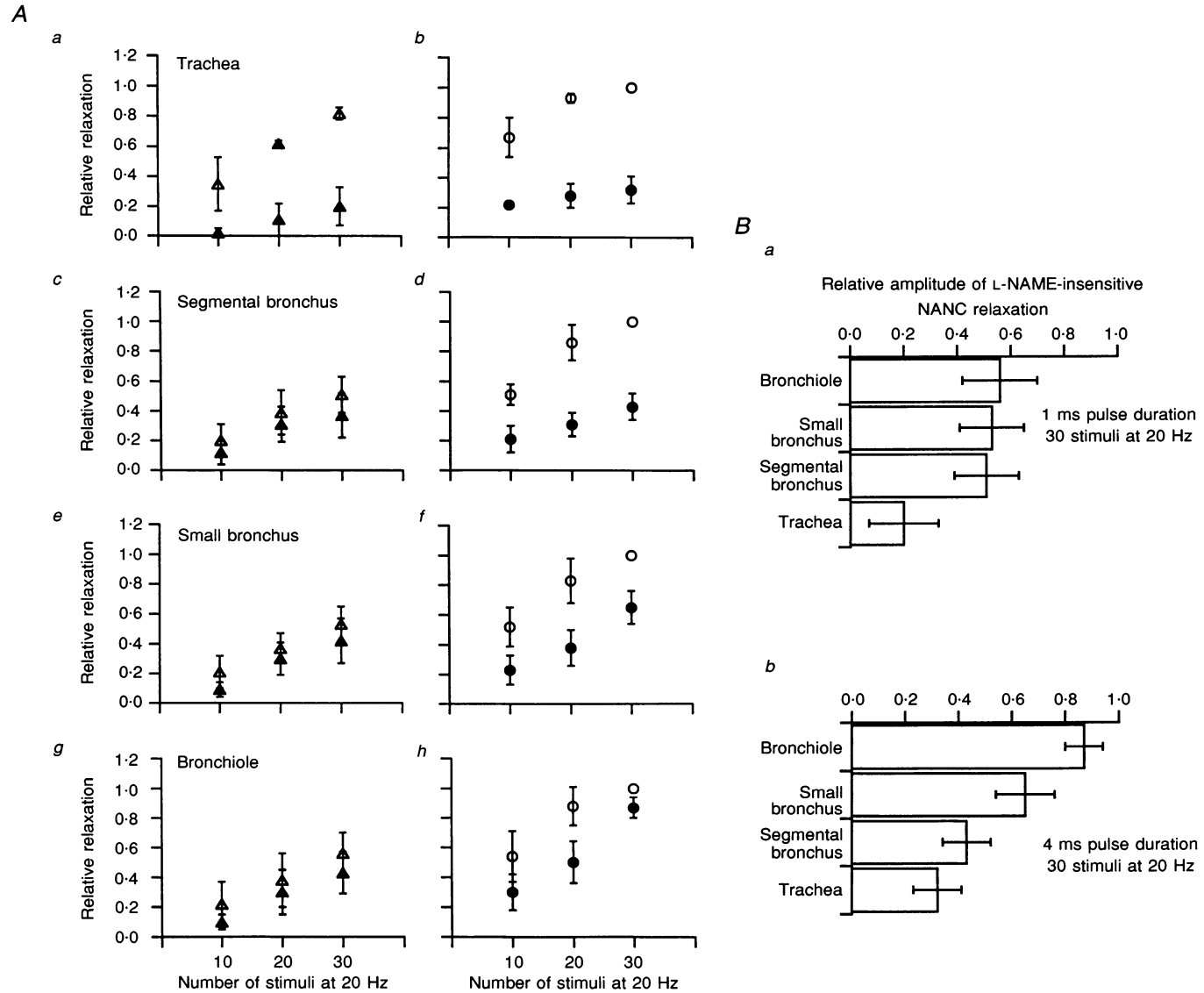

Figure 3. Effects of L-NAME (10^{-5} M) on EFS-induced NANC relaxations

A, relationship between the relative amplitude of NANC relaxation and number of stimuli at 20 Hz, where the amplitude of NANC relaxation evoked by 30 stimuli at 20 Hz with 4 ms pulse duration is taken as a relative relaxation of 1·0. Repetitive field stimulation of 10, 20 and 30 stimuli at 20 Hz with 1 ms pulse duration (△,▲) or 10, 20 and 30 stimuli at 20 Hz with 4 ms pulse duration (○,●) in the absence (△,○) or presence (▲,●) of L-NAME (10^{-5} M) during the contraction evoked by 5-HT (10^{-5} M) in the presence of atropine and guanethidine (10^{-6} M each). *B*, relative amplitude of L-NAME-insensitive NANC relaxation evoked by 1 ms (*Ba*) or 4 ms (*Bb*) pulse duration in central and peripheral airway, where the amplitude of NANC relaxation evoked by 30 stimuli at 20 Hz with 4 ms pulse duration is taken as a relative relaxation of 1·0. Bars indicate 2 × S.D.

activation of the first and L-NAME-sensitive component is lower than the other component. However, the difference in the threshold is small and when the pulse duration of 1 ms was used, 5 stimuli at 20 Hz evoked initial and second components of the NANC relaxation (data not shown).

Figure 4*B* shows the relationship between the number of stimuli and relative amplitude of the first and second component of NANC relaxation in the presence or absence of L-NAME observed in small bronchi and bronchioles, where the maximum relaxation induced by thirty stimuli at 20 Hz with 4 ms pulse duration was taken as a relative amplitude of 1·0. L-NAME (10^{-5} M) completely suppressed the first component of the NANC relaxation evoked by repetive stimuli with 1 and 4 ms pulse duration, but showed practically no effect on the second component in bronchioles.

EFS-induced NANC relaxation in peripheral airway of cat pretreated with L-NAME

To establish further the contribution of NO to the NANC relaxation induced by EFS in the peripheral airway of the cat, experiments were conducted using small bronchi from L-NAME-pretreated cats. As was expected, EFS applied during contraction of the bronchus evoked by 5-HT (10^{-5} M) in the presence of atropine and guanethidine (10^{-6} M each) did not produce the characteristic biphasic NANC relaxation obtained with small bronchus of normal cat. Figure 5*a* shows an example of EFS-induced NANC relaxation where ten, twenty and thirty stimuli at 20 Hz with 4 ms pulse duration were applied to the bronchus of L-NAME-pretreated cat. The NANC relaxation produced by EFS showed only the slow component and the presence of L-NAME (10^{-5} M) in the perfusing solution did not alter

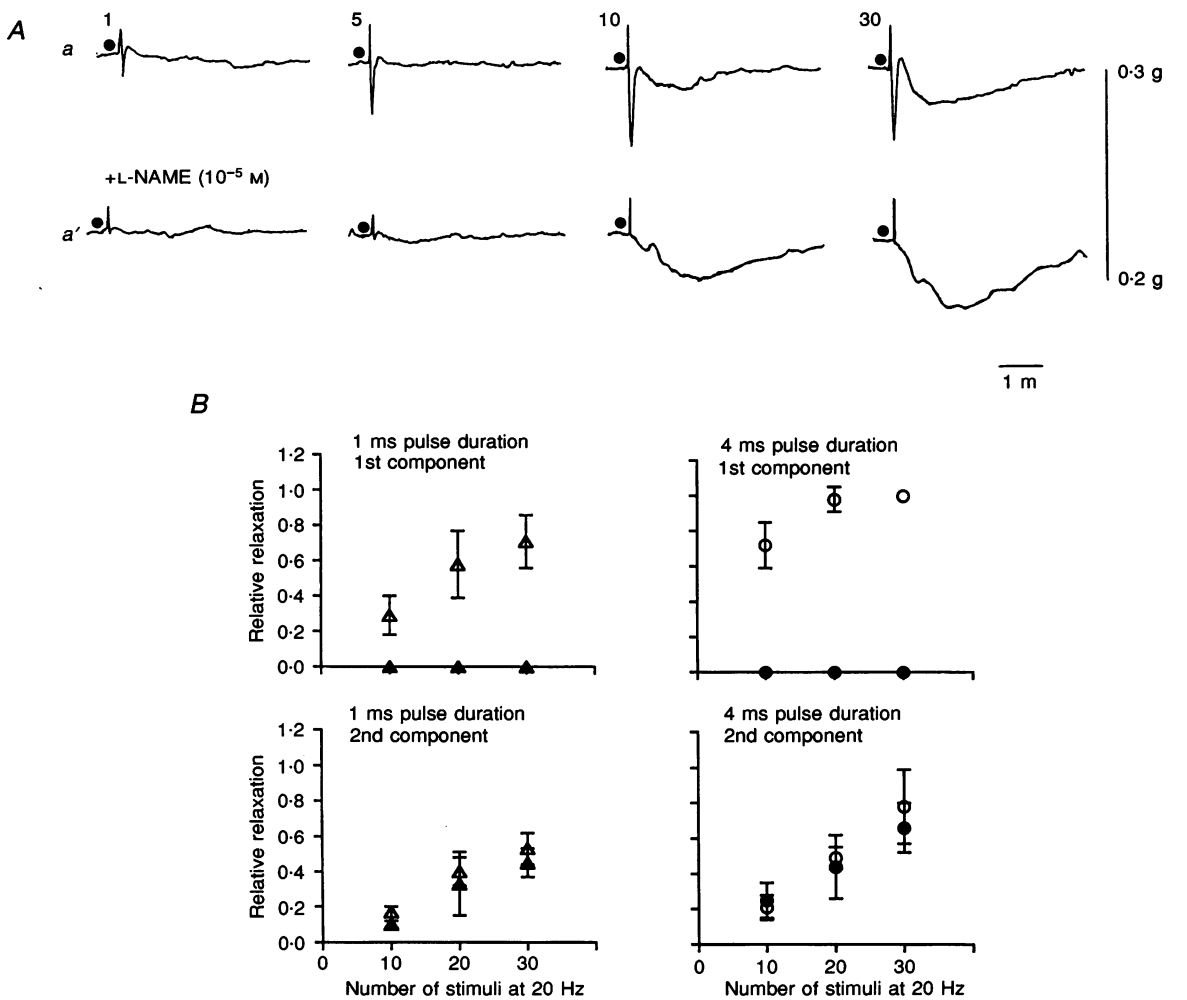

Figure 4. Effects of EFS with short pulse duration (600 μs) on bronchiole

Aa and *a'*, single or repetitive field stimulation (●; 5, 10 and 30 stimuli) at 20 Hz with 600 μs pulse duration was applied during the contraction of bronchioles evoked by 5-HT (10^{-5} M) in the presence of atropine and guanethidine (10^{-6} M each) in the absence (*a*) or presence (*a'*) of L-NAME (10^{-5} M). *B*, effects of L-NAME (10^{-5} M) (▲,●) on the 1st and 2nd component of NANC relaxations evoked by repetitive field stimulation with 1 ms pulse duration (△) or 4 ms pulse duration (○) in the small bronchi and bronchioles. Bars indicate 2 × S.D.

the profile of the relaxation. Figure 5*b* shows the characteristic biphasic NANC relaxation induced by EFS (10, 20, 30 stimuli at 20 Hz) in the bronchus of control cat pretreated with vehicle only. Addition of L-NAME (10^{-5} M) to the perfusing solution abolished the initial fast component.

Effects of VIP antagonist on L-NAME-insensitive NANC relaxation

To study the generating mechanisms involved in the L-NAME-insensitive second component of NANC relaxation in small bronchi and bronchioles, we observed the effects of VIP antagonist (Gozes, Meltzer, Rubinbout, Brenneman & Fridkin, 1989) on the NANC relaxation in the presence of L-NAME (10^{-5} M). Figure 6*A* shows an example of the effect of VIP antagonist (10^{-6} M) on the L-NAME-insensitive NANC relaxation. After pretreatment of the tissue with L-NAME (10^{-5} M) for 1 h, EFS (30 stimuli at 20 Hz with 4 ms pulse duration) was applied every 20 min. The amplitude of the L-NAME-insensitive NANC relaxation showed a slight fade phenomenon during the first 1 h of stimulation at an interval of every 20 min. Application of VIP antagonist (10^{-6} M) enhanced the rate of decrease in the amplitude of L-NAME-insensitive NANC relaxation, and after 1 h the amplitude decreased to about 50% of the control value. Figure 6*B* shows the time course of the effects of VIP antagonist on the L-NAME-insensitive NANC relaxations observed with nine preparations.

Effects of α-chymotrypsin on L-NAME-insensitive NANC relaxation

We also observed the effects of α-chymotrypsin on the L-NAME-insensitive second component of the NANC relaxations after pretreatment of the tissues with L-NAME (10^{-5} M). Application of α-chymotrypsin (1 U ml^{-1}) progressively reduced the muscle tone evoked by 5-HT of the ring preparations of bronchi and bronchioles and therefore short periods of incubation (30 min) were used. The amplitude of NANC relaxations were evaluated in relation to the degree of muscle tension at any given time to exclude interferences of the spontaneous decrease in tone. α-Chymotrypsin suppressed the amplitude of the L-NAME-insensitive component of the NANC relaxation to 40–50% of the original value within 30 min. Figure 7 shows the effects of α-chymotrypsin on the relative amplitude of L-NAME-insensitive NANC relaxation evoked by ten, twenty or thirty stimuli at 20 Hz with 4 ms pulse duration. α-Chymotrypsin reduced the L-NAME-insensitive NANC relaxations induced by ten, twenty or thirty stimuli at 20 Hz to similar extents.

Effects of EFS on the resting membrane potential of the airway smooth muscle cells of the bronchi and bronchioles

To observe the effects of EFS on the resting membrane potential of the airway smooth muscle cells, we used a microelectrode. The mean value of the resting membrane

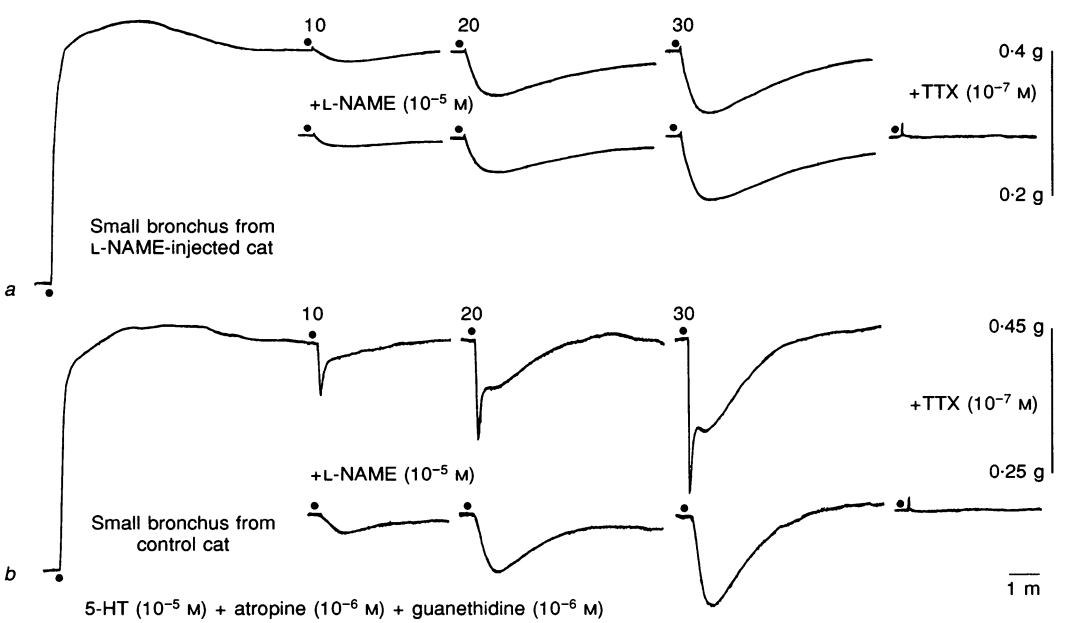

Figure 5. Effects of pretreatment of cat with L-NAME on NANC relaxation

Effects of L-NAME (10^{-5} M) on NANC relaxation induced in small bronchus from L-NAME-pretreated cat. Repetitive field stimulation of 10, 20 and 30 stimuli at 20 Hz with 4 ms pulse duration (●) evoked mono- and biphasic NANC relaxation in small bronchi obtained from L-NAME-pretreated (*a*) and control (pretreated with vehicle only; *b*) cats, respectively. L-NAME (10^{-5} M) selectively abolished the initial fast NANC relaxation in control bronchus. L-NAME-insensitive NANC relaxations were abolished by TTX (10^{-7} M).

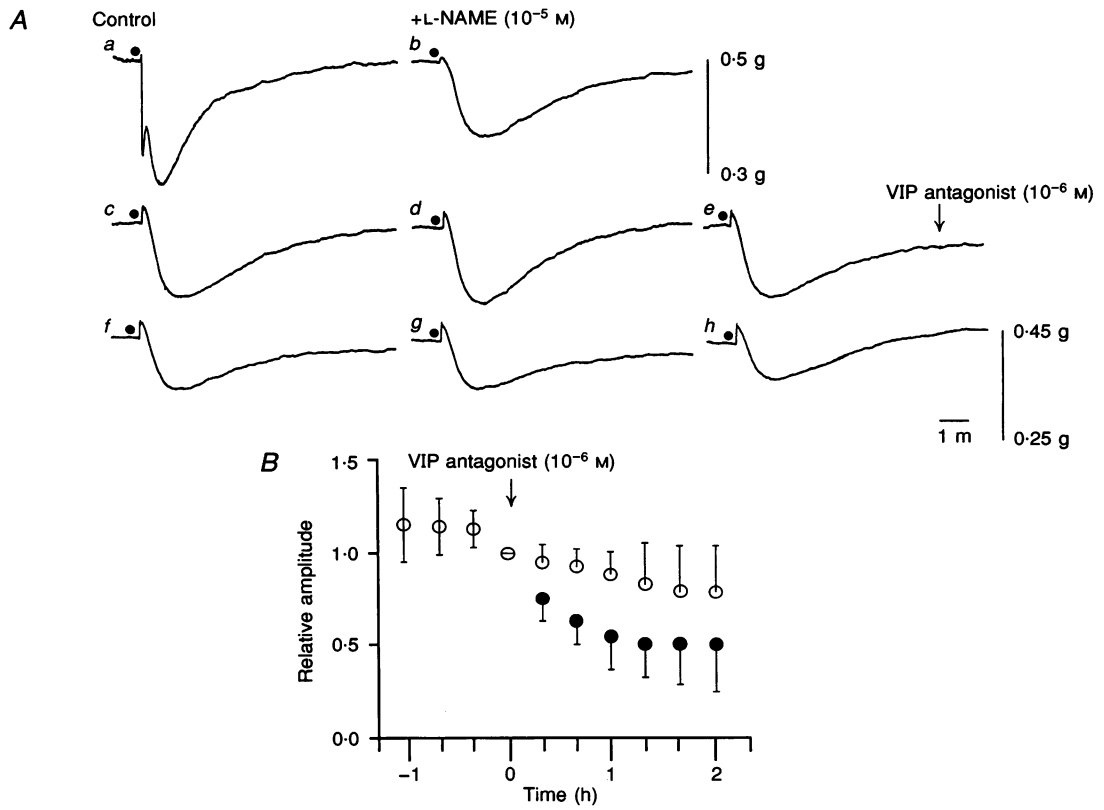

Figure 6. Effects of VIP antagonist (10^{-6} M) on L-NAME-insensitive NANC relaxation

Aa, electrical field stimulation of 30 stimuli at 20 Hz with 4 ms pulse duration applied every 20 ms evoked biphasic NANC relaxations during contraction evoked by 5-HT (10^{-5} M) in small bronchus. *Ab–e*, L-NAME (10^{-5} M) selectively abolished the first component of the relaxations, the amplitude of which gradually decreased during the perfusion with L-NAME-containing Krebs solution. *Af–h*, application of VIP antagonist (10^{-6} M) enhanced the decrease in the amplitude of L-NAME-insensitive NANC relaxation. *B*, changes in the relative amplitude of L-NAME-insensitive NANC relaxation in the absence (○) and presence (●) of VIP antagonist, where the amplitude of L-NAME-insensitive NANC relaxation just before application of VIP antagonist is taken as a relative amplitude of 1·0. Bars indicate 2 × S.D.

potential of the airway smooth muscle differed in the central and peripheral airway. Namely, the mean values were −69·2 ± 1·1 (± S.D., $n = 18$), −71·8 ± 1·3 (± S.D., $n = 13$), −74·5 ± 1·4 (± S.D., $n = 13$) and −78·7 ± 1·5 (± S.D., $n = 8$) in the trachea, segmental bronchi, small bronchi and bronchioles, respectively.

EFS (single or repetitive stimuli at 20 Hz) evoked excitatory junction potentials (EJPs) in all regions of the airway examined. Atropine (10^{-6} M) completely abolished the generation of EJPs, and, in the presence of atropine and guanethidine (10^{-6} M each), EFS (single or repetitive stimuli) evoked no change in the membrane potential of the

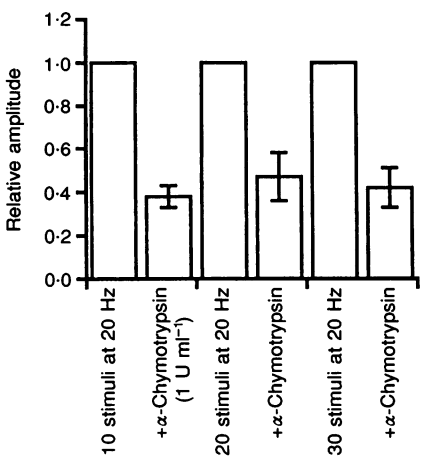

Figure 7. Effects of α-chymotrypsin on L-NAME-insensitive NANC relaxation in small bronchi and bronchioles

Electrical field stimulation of 10, 20 and 30 stimuli with 4 ms pulse duration was applied in the presence of L-NAME (10^{-5} M) to evoke L-NAME-insensitive NANC relaxations. The relative amplitudes of NANC relaxations 30 min after application of α-chymotrypsin (1 U ml^{-1}) relative to the control value at each stimuli condition are shown. Bars indicate 2 × S.D.

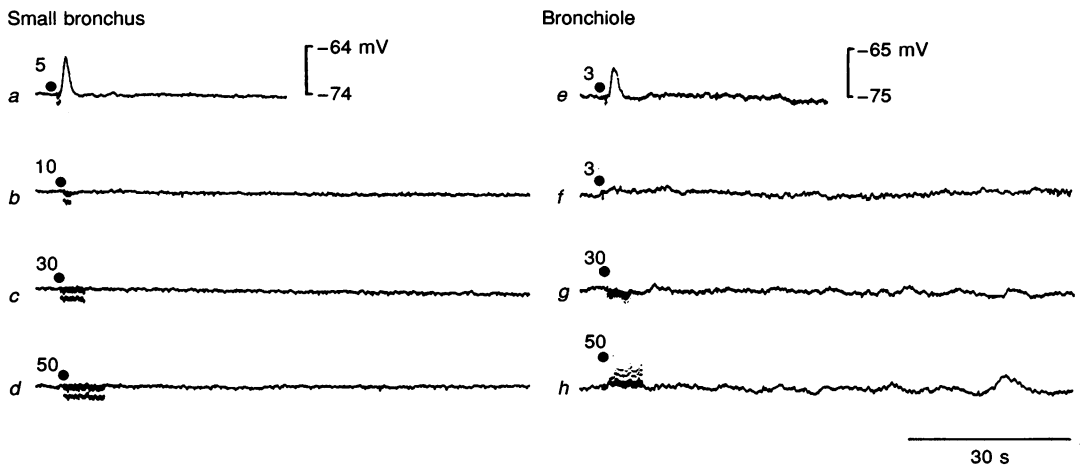

Figure 8. Effects of EFS on resting membrane potential of smooth muscle cell of bronchus and bronchiole

a and *e*, traces of EJPs evoked by repetitive stimuli (●) recorded from small bronchus and bronchiole in control condition. *b–d* and *f–h*, after application of atropine and guanethidine (10^{-6} M each), EFS did not alter the resting membrane potential of the smooth muscle cells in the bronchus and bronchiole.

airway smooth muscle cells. Figure 8 shows an example, where the microelectrode was kept inside the smooth muscle cells in bronchus and bronchiole throughout the experiment, before and after application of atropine and during application of repetitive EFS. Repetitive stimuli at 20 Hz (3–50 stimuli) did not evoke any change in the resting membrane potential. This means that EFS induces L-NAME-sensitive and -insensitive NANC relaxations without changing the membrane potential of the airway smooth muscle cells.

DISCUSSION

We demonstrated that EFS with short and longer pulse duration evokes NANC relaxation in the cat airway from trachea to bronchioles, when the tissues were incubated with atropine and guanethidine (10^{-6} M each) and were precontracted with 5-HT (10^{-5} M). The relative amplitude of the NANC relaxations in trachea, bronchi and bronchioles were similar. In contrast, in equine airway the amplitude of NANC relaxation decreased from the trachea to bronchi and eventually disappeared in 5 mm o.d. bronchi, indicating the lack of functional i-NANC innervations in small bronchi and bronchioles in this animal species. Since strong adrenergic inhibitory relaxation can be detected throughout calf airways under the same experimental conditions, the authors concluded that the lack of inhibitory innervation in the small airways in the horse was not due to inappropriate experimental procedures. This may indicate that the functional inhibitory innervations diminish along the tracheobronchial tree in equine airway (Yu *et al.* 1994). However, the present experiments show that in cat airway the functional inhibitory innervations are present in the small airways including bronchi and bronchioles, suggesting species specificity.

Previously we reported that L-NNA or L-NAME greatly suppressed the EFS-induced NANC relaxation in the cat trachea but did not abolish NANC relaxation induced by EFS, especially when a longer pulse duration (4 ms) was applied at high frequency (20 Hz). A substantial part of L-NNA-resistant muscle relaxation was sensitive to tetrodotoxin, thereby indicating that the L-NNA-resistant relaxation was neurogenic in origin (Jing *et al.* 1995). Similarly, in the present experiments L-NAME (10^{-4}–10^{-5} M) greatly reduced the amplitude of the NANC relaxation in the trachea, but did not abolish it. We also demonstrated that EFS induces biphasic NANC relaxations with an initial fast and a second slow component in the small bronchi and bronchioles. L-NAME completely abolished the initial fast component without affecting the second slow component, indicating that the NANC relaxation in the cat bronchi and bronchioles is due to release of at least two different neurotransmitters and that NO, or a NO-containing compound, is involved in the initial fast component. In parallel, NANC relaxation induced by EFS in the small bronchi of L-NAME-pretreated cat was also devoid of the initial fast component. This observation lends further support to the evidence that the initial fast component of NANC relaxation in the peripheral airway of the cat is due to NO or NO-containing compounds. The roles of the two inhibitory neurotransmitters may differ in the peripheral airways, namely one which induces fast relaxation with short delay after the activation of NANC nerves and the other which evokes long-lasting relaxation with relatively long delay. Therefore, it seems reasonable to assume that the latter component is important for the long-lasting airway relaxation in the periphery. The amplitude of L-NAME-insensitive component of the NANC relaxation was partially suppressed by VIP

antagonists and α-chymotrypsin, indicating that VIP may be involved in L-NAME-insensitive NANC relaxation at least partially. These observations taken together indicate that two different components are involved in the NANC relaxation in the cat airway including trachea, where the time course of the NANC relaxation is monophasic.

Though the neurotransmitter(s) responsible for the NANC relaxation in the airway have not been conclusively identified, NO or NO-containing compounds and VIP have emerged as strong candidates for this role. NO or the NO donors such as nitrosothiol compounds (Myers, Minor, Guerra, Bates & Harrison, 1990) may be involved as a NANC inhibitory neurotransmitter in the relaxation of airway smooth muscles since: (i) NO is produced in neural tissue as a product of the action of NO synthase (Bredt, Hwang & Snyder, 1990); (ii) blocking NO synthase inhibited the formation of NO as well as NANC relaxation induced by EFS in the trachea of guinea-pig (Tucker *et al.* 1990; Li & Rand, 1991), pig (Kannan & Johnson, 1992), cat (Fisher *et al.* 1993) and man (Belvisi *et al.* 1992; Ellis & Undem, 1992) and (iii) NO applied exogenously into the airway mimics the effect of inhibitory nerve stimulation on the pulmonary resistance (Dupy, Shore, Drasen, Frostell, Hill & Zapol, 1992). The present study also shows that the NO synthase inhibitor L-NAME suppresses the amplitude of NANC relaxation in trachea and selectively abolished the initial fast component of the NANC relaxations in bronchi and bronchioles, indicating that NO or a NO-containing compound is involved in the NANC relaxation in the central and peripheral airway of the cat.

It remains to be determined whether NO itself or an NO-containing compound such as a nitrosothiol compound (Myers *et al.* 1990) is the inhibitory neurotransmitter. Nitrosothiol compounds are less sensitive than NO to inactivation by superoxide anions (O_2^-) and to inhibition by methoxyhaemoglobin (HbO_2; Furchgott, Jothianandan & Khan, 1992). It was reported that Methylene Blue (MB) and HbO_2 each shifted the dose–response curve for NO to the right without affecting EFS-induced NANC relaxation in sheep urethral muscle. Similarly dose-dependent responses to *S*-nitroso-L-cysteine (NC) were not affected by MB. The inhibition of relaxation to NO by MB was prevented by superoxide dismutases, suggesting that the inhibition was caused by extracellular generation of superoxide anions (García-Pascual & Triguero, 1994). Similarly, in the cat trachea, we reported that MB and HbO_2 are much less potent than L-NAME in suppressing the amplitude of NANC relaxation. It is known that nitrosothiols can supply NO to the cytoplasm after denitrosation at the external smooth muscle membranes (Kowaluk & Fung, 1990). These observations may support the suggestion that NO donors such as nitrosothiol compound may be the neurotransmitter.

On the other hand, immunofluorescence techniques revealed the presence of VIP-immunoreactive nerve fibres in airway smooth muscle layers of the cat (Hakanson, Sundler, Moghimzaden & Leander, 1983) and VIP immunoreactivity has been localized to cholinergic nerves (Lundberg, 1981). Furthermore, VIP is released during electrical field stimulation of guinea-pig trachea and a correlation exists between the amount of VIP release and degree of relaxation induced by EFS (Matsuzaki *et al.* 1980). Incubation with VIP antiserum or immunization to VIP reduces the tracheal relaxation in response to EFS (Matsuzaki *et al.* 1980; Hakoda, Xie, Aizawa, Inoue, Hirata & Ito, 1991). All these data taken together suggest that VIP is also involved in NANC relaxations in the airway. In the present study, the amplitude of L-NAME-insensitive components of the NANC relaxation in bronchi and bronchioles was suppressed by VIP antagonist or α-chymotrypsin. These observations also indicate that VIP is involved in the NANC relaxations in the cat airway. However, VIP antagonists such as [Ac-Tyr^1,D-Phe^2]-GRF (1–29)-NH_2 and [4-Cl-D-Phe^6,Leu^{17}]-VIP or a newly synthesized VIP antagonist (Gozes *et al.* 1989) only partially suppressed the amplitude of NANC relaxation in the presence of L-NAME in the trachea (Jing *et al.* 1995) and bronchus (present observations), suggesting that neurotransmitter(s) other than VIP may be involved in the L-NAME-insensitive NANC relaxation.

One feature of NANC-inhibitory neurotransmission in the cat trachea is that activation of the inhibitory nerves causes muscle relaxation without affecting the resting membrane potential or input membrane resistance of the tracheal smooth muscle, i.e. through pharmaco–mechanical coupling (Ito & Takeda, 1982). In the present experiments, we confirmed that activation of NANC nerves in the bronchiole evokes no change in the resting membrane potential and induces relaxation. Cys-NO (10^{-9}–10^{-5} M) or VIP (10^{-9}–10^{-7} M) evoke muscle relaxation without changing the resting membrane potential, and these mimic the postjunctional membrane response of the cat trachea to activation of NANC inhibitory nerves by EFS (Jing *et al.* 1995).

It remains to be established whether a single functional class of inhibitory neurones releases two inhibitory neurotransmitters at the same time, or two functionally distinct classes of inhibitory neurones each release one inhibitory neurotransmitter preferentially in the cat airway.

It was suggested that NANC relaxation in cat trachea may be classified into two different components according to the threshold for activation of the relaxation (Jing *et al.* 1995). In fact, in the bronchioles, single EFS with short pulse duration (600 μs to 1 ms) evoked initial fast and L-NAME-sensitive relaxation, but did not evoke the second

component of the NANC relaxations, indicating that the threshold for activation of the initial component is lower than that of the second component. However, when repetitive stimuli (> 5) at 20 Hz were applied, the initial and second components of the NANC relaxations were triggered even when pulses with short duration (600 μs to 1 ms) were used. Thus, practically, it is not feasible to distinguish the two components of the NANC relaxations according to the threshold for activations.

In conclusion, we have demonstrated that at least two neurotransmitters, possibly NO or NO-containing compounds and VIP, are involved in the inhibitory NANC neurotransmissions in the airway of the cat and that the distribution profile of the two components differs in the central and peripheral airway.

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